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(54) Title: NOVEL OPIOID PEPTIDE INHIBITORS			
(57) Abstract This invention provides novel peptides having the ability to inhibit binding of the mu specific ligand ³ H-[D-Ala ² , MePhe ⁴ , Gly-ol ⁵] enkephalin ("DAGO") to the opioid receptors in crude rat brain homogenate.			

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NOVEL OPIOID PEPTIDE INHIBITORS

BACKGROUND OF THE DISCLOSURE

This invention generally relates to novel peptides having the ability to inhibit ligand binding to an
5 opioid receptor.

There are at least three known subtypes of opioid receptors, mu, kappa, and delta; with some evidence for two additional receptor subtypes. The use of synthetic peptides has been instrumental in the delineation of these
10 subtypes and for providing analogues that can be used for studying the interactions of ligands specific to these receptor systems in both in vitro and in vivo systems.

Recent advances in methods for the preparation
15 and screening of a large numbers of individual peptides has enabled a large number of peptides to be used in all areas of biomedical research, including research regarding the interaction of a ligand to the opiate receptor. Even with these advances, however, basic research and drug discovery
20 has been limited by the availability of the requisite large number of diverse opiate agonists and antagonists required to ascertain the relationship between a ligand for a particular opiate receptor subtype. Thus, a need exists for large numbers of individual peptides for use in
25 biomedical research, including those for the study of opiate ligand-receptor interactions. This invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

30 This invention provides novel peptides having the ability to inhibit ^3H -[D-Ala², MePhe⁴, Gly-ol⁵]enkephalin ("DAGO") binding to opioid receptors in crude rat brain homogenates. The novel peptides fall within four general structures: Ac-L-Arg-L-Phe-L-Met-L-Trp-L-Met-L-Thr-L-Xaa-NH₂,

(SEQ ID NO: 1); Ac-D-Arg-D-Phe-D-Trp-D-Trp-D-Gly-D-Xaa-NH₂
(SEQ ID NO: 2); Ac-D-Arg-D-Phe-D-Trp-D-Ile-D-Asn-D-Xaa-NH₂
(SEQ ID NO: 3); and Ac-D-Arg-D-Phe-D-Trp-D-Met-D-Tyr-D-Xaa-
NH₂ (SEQ ID NO: 4). Within each genus, Xaa is substituted
5 by a specific amino acid.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A through 1D graphically depict the ability of each peptide to inhibit binding of [³H]-DAGO to the μ receptor as measured by the radio-receptor assay. In
10 these figures, "o" is the equivalent of the amino acid code "Xaa".

DETAILED DESCRIPTION OF THE INVENTION

This invention provides peptides useful as inhibitors of ³H-[D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin ("DAGO")
15 which is known to bind with high selectivity to the μ ("mu") opioid receptor subtype. Each of the peptides has an acetyl group ("Ac") at the N-terminus and an amide group ("NH₂") on the C-terminus. In one embodiment, the peptide has the structure Ac-L-Arg-L-Phe-L-Met-L-Trp-L-Met-L-Thr-L-
20 Xaa-NH₂, wherein Xaa is an L-amino acid such as L-Lys, L-Arg, L-Thr, L-Ser, L-Gln, L-Pro, L-His, L-Ala, L-Asn, L-Met, L-Gly, L-Leu, L-Tyr, L-Val, L-Trp, L-Asp, L-Cys, L-Glu, L-Phe, and L-Ile. In another embodiment, the peptide is described by the formula Ac-D-Arg-D-Phe-D-Trp-D-Trp-D-
25 Gly-D-Xaa-NH₂, wherein D-Xaa is D-Leu, D-Met, D-Val, D-Trp, D-Lys, D-Tyr, D-Arg, D-Ile, D-Pro, D-Phe, D-His, D-Ser, D-Cys, D-Asn, D-Thr, D-Gln, D-Ala, D-Gly, D-Asp, or D-Glu. Also within the scope of this invention is a peptide having the structure Ac-D-Arg-D-Phe-D-Trp-D-Ile-D-Asn-D-Xaa-NH₂,
30 wherein D-Xaa is a D-amino acid such as D-Lys, D-Ala, D-Arg, D-Gln, D-Pro, D-Asn, D-Ser, D-Tyr, D-Met, D-Gly, D-Thr, D-His, D-Trp, D-Leu, D-Phe, D-Glu, D-Cys, D-Val, D-Asp and D-Ile. Further provided by this invention is a peptide

having the structure Ac-D-Arg-D-Phe-D-Trp-D-Met-D-Tyr-D-Xaa-NH₂, wherein D-Xaa is a D-amino acid such as D-Arg, D-Lys, D-His, D-Ser, D-Thr, D-Gln, D-Pro, D-Ala, D-Asn, D-Gly, D-Tyr, D-Leu, D-Met, D-Phe, D-Cys, D-Trp, D-Glu, D-Asp, D-Val, or D-Ile.

One skilled in the art, using the above formulae, can easily reproduce the peptides of this invention by synthesis on an automated peptide synthesizer (Model 430A, Applied Biosystem, Foster City, California USA) utilizing the directions provided by the manufacturer. After manufacture, the peptides are assayed for receptor binding activity using the radio-receptor assay outlined below. Because these peptides bind to the μ and other receptor subtypes, they can be used in in vitro assays to study the opiate receptor subtypes. For example, in a sample receptor of unknown type or origin, the peptides, after being labeled with a detectable marker such as a radioisotope, can be contacted with the receptor sample under conditions which specifically favor a particular receptor subtype binding to the peptide(s). Unbound receptor and peptide can be removed, for example, by washing with a saline solution, and bound receptor can then be detected using methods well known to those skilled in the art.

In addition to the peptides utility in in vitro screening method for assaying organic compounds having specificity for the opioid receptors, the peptides also are useful as drugs to treat pathologies associated with other compounds which interact with the opioid receptor system. It can be envisioned that these peptides can be used for therapeutic purposes to block the peripheral effects of existing centrally acting pain killers such as morphine. Since it is known that the majority of peptides do not readily cross the blood-brain barrier (and therefore elicit no central effect), and since morphine has a number of

deleterious effects in the periphery which are not required for the desired analgesic effects, it can be anticipated that the subject peptides may have value in blocking the constipation and pruritis (itching) associated with morphine. The novel peptides claimed can be incorporated into pharmaceutical compositions. The pharmaceutical composition is prepared by combining the peptide with a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents.

Methods of administering a pharmaceutical are well known in the art and include but are not limited to administration orally, intravenously, intramuscularly or intraperitoneal. Administration can be effected continuously or intermittently and will vary with the subject and is dependent on the type of treatment and potency of the peptide used.

Radio-receptor assay

Crude membrane homogenates were prepared using a modification of the method described by Paternak, G.W. et al., Mol. Pharmacol., 11:340-351 (1975), incorporated herein by reference. Rat brains frozen in liquid nitrogen were obtained from Rockland, Inc. (Gilberstville, PA). The brains were thawed the cerebella removed, and the remaining tissue weighed. Each brain was individually homogenized in 40 ml Tris-HCl buffer (50mM, pH 7.4, 4°C) and centrifuged (Sorvall RC5C SA-600 16,000 rpm) for 10 minutes. The pellets were resuspended in fresh Tris-HCl buffer and incubated at 37°C for 40 minutes. Following incubation, the suspensions were centrifuged as before, the resulting

pellets resuspended in 100 volumes of Tris buffer, and the suspensions combined. Membrane suspensions were prepared and used in the same day. Protein content of the crude homogenates ranged from 0.15 to 0.2 mg/ml as determined using the method described by Bradford, M.M. Anal. Biochem. 72:248-254 (1976), incorporated herein by reference.

Binding assays were carried out in polypropylene tubes. Each tube contained 0.5 ml of membrane suspension, 8nM [3 H]-DAGO (specific activity 36Ci/mmol, 160,000 cpm), 0.08 mg/ml peptide mixture and Tris-HCl buffer in a total volume of 0.65 ml. Assay tubes were incubated for 60 minutes at 25°C. The reaction was terminated by filtration through GF-B filters. The filters were subsequently washed with 6 ml Tris-HCl buffer, at 4°C. Bound radioactivity was counted on an LKB Beta-plate Liquid Scintillation Counter and expressed in counts per minute (cpm). Inter- and intra-assay variation standard curves were determined by incubation of [3 H]-DAGO in the presence of a range of concentrations of unlabeled DAGO (0.13-3900nM). Both the tritiated and non-tritiated forms of DAGO were obtained from the National Institute of Drug Abuse (NIDA) repository, as prepared by Multiple Peptide Systems (San Diego, CA). A control curve was included on each plate for each assay (using a 96-well format). Competitive inhibition assays were performed as above using serial dilutions of the peptide mixture. IC₅₀ values (the concentration necessary to inhibit 50% of [3 H]-DAGO binding) were then calculated using the software GRAPHPAD (ISI, San Diego, CA) and were found to be consistent in three determinations. The average values for each peptide are presented in Figure 1.

Although the invention has been described with reference to the disclosed embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly,

the invention is limited only by the following claims.

What is claimed is:

1. A peptide having the structure:

Ac-L-Arg-L-Phe-L-Met-L-Trp-L-Met-L-Thr-L-Xaa-NH₂;

5 wherein L-Xaa is an L-amino acid selected from the group consisting of L-Lys, L-Arg, L-Thr, L-Ser, L-Gln, L-Pro, L-His, L-Ala, L-Asn, L-Met, L-Gly, L-Leu, L-Tyr, L-Val, L-Trp, L-Asp, L-Cys, L-Glu, L-Phe, and L-Ile.

2. A peptide having the structure:

10 Ac-D-Arg-D-Phe-D-Trp-D-Trp-D-Gly-D-Xaa-NH₂;

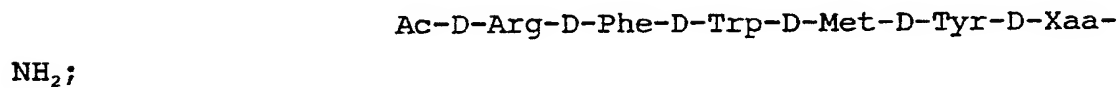
wherein D-Xaa is a D-amino acid selected from the group consisting of D-Leu, D-Met, D-Val, D-Trp, D-Lys, D-Tyr, D-Arg, D-Ile, D-Pro, D-Phe, D-His, D-Ser, D-Cys, D-Asn, D-Thr, D-Gln, D-Ala, D-Gly, D-Asp, and D-Glu.

- 15 3. A peptide having the structure:

Ac-D-Arg-D-Phe-D-Trp-D-Ile-D-Asn-D-Xaa-NH₂;

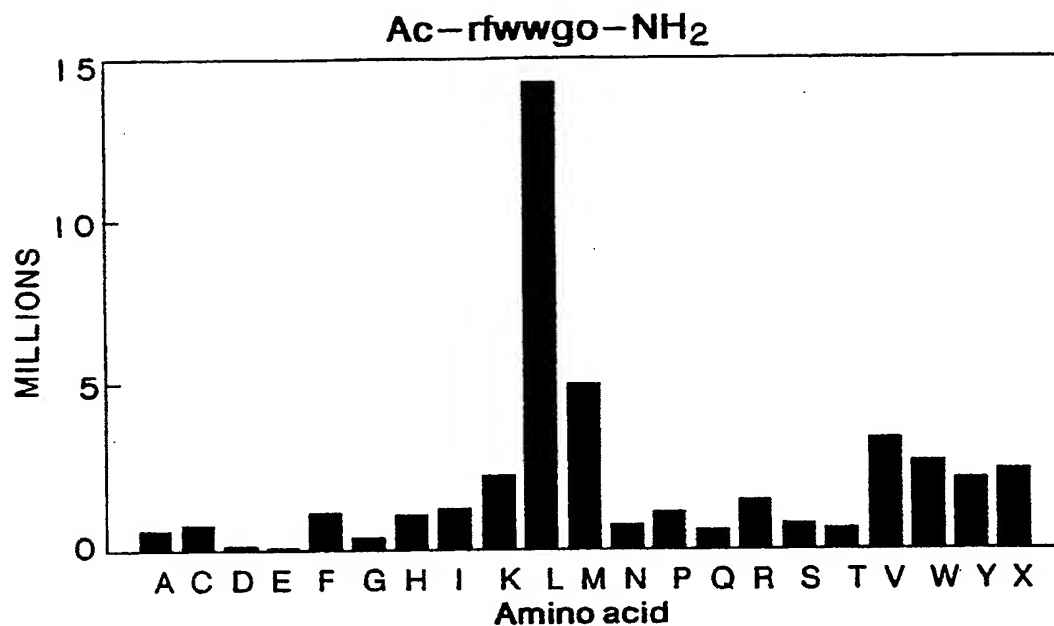
20 wherein D-Xaa is a D-amino acid selected from the group consisting of D-Lys, D-Ala, D-Arg, D-Gln, D-Pro, D-Asn, D-Ser, D-Tyr, D-Met, D-Gly, D-Thr, D-His, D-Trp, D-Leu, D-Phe, D-Glu, D-Cys, D-Val, D-Asp and D-Ile.

4. A peptide having the structure:



5 wherein D-Xaa is a D-amino acid selected from the group consisting of D-Arg, D-Lys, D-His, D-Ser, D-Thr, D-Gln, D-Pro, D-Ala, D-Asn, D-Gly, D-Tyr, D-Leu, D-Met, D-Phe, D-Cys, D-Trp, D-Glu, D-Asp, D-Val, and D-Ile.

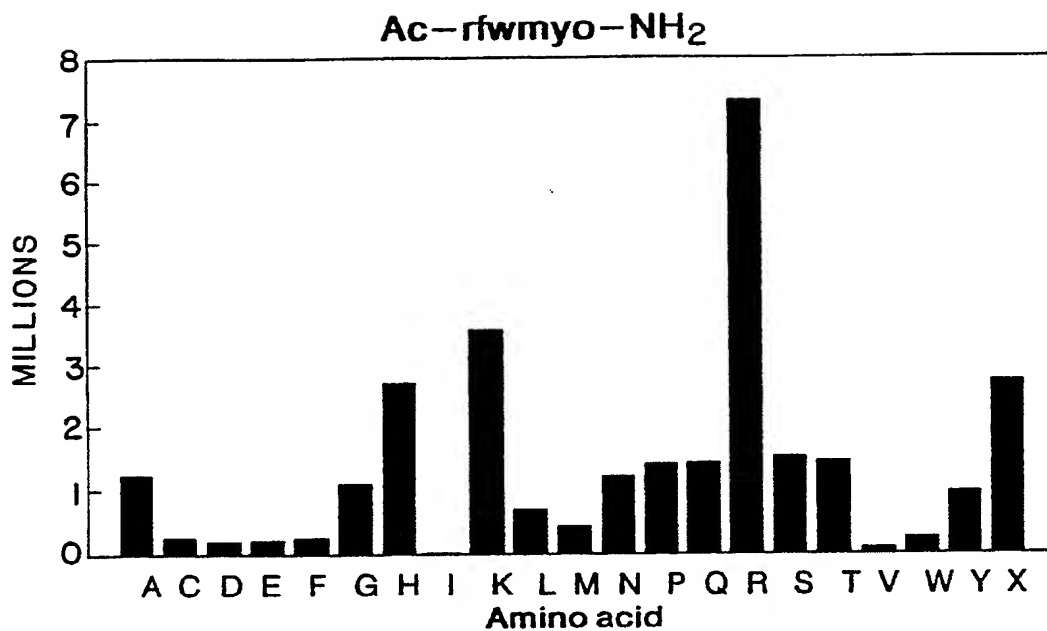
1 / 4

**Ac-rfwwgo-NH₂**

	nM
Ac-rfwwgl	70
Ac-rfwwgm	201
Ac-rfwwgv	298
Ac-rfwwgw	377
Ac-rfwwgx	419
Ac-rfwwgk	451
Ac-rfwwgy	470
Ac-rfwwgr	671
Ac-rfwwgi	809
Ac-rfwwgp	871
Ac-rfwwgf	885
Ac-rfwwgh	923
Ac-rfwwgs	1283
Ac-rfwwgc	1295
Ac-rfwwgn	1370
Ac-rfwwgt	1584
Ac-rfwwgq	1665
Ac-rfwwga	1698
Ac-rfwwgg	2573
Ac-rfwwgd	8652
Ac-rfwwge	14855

FIG. 1A

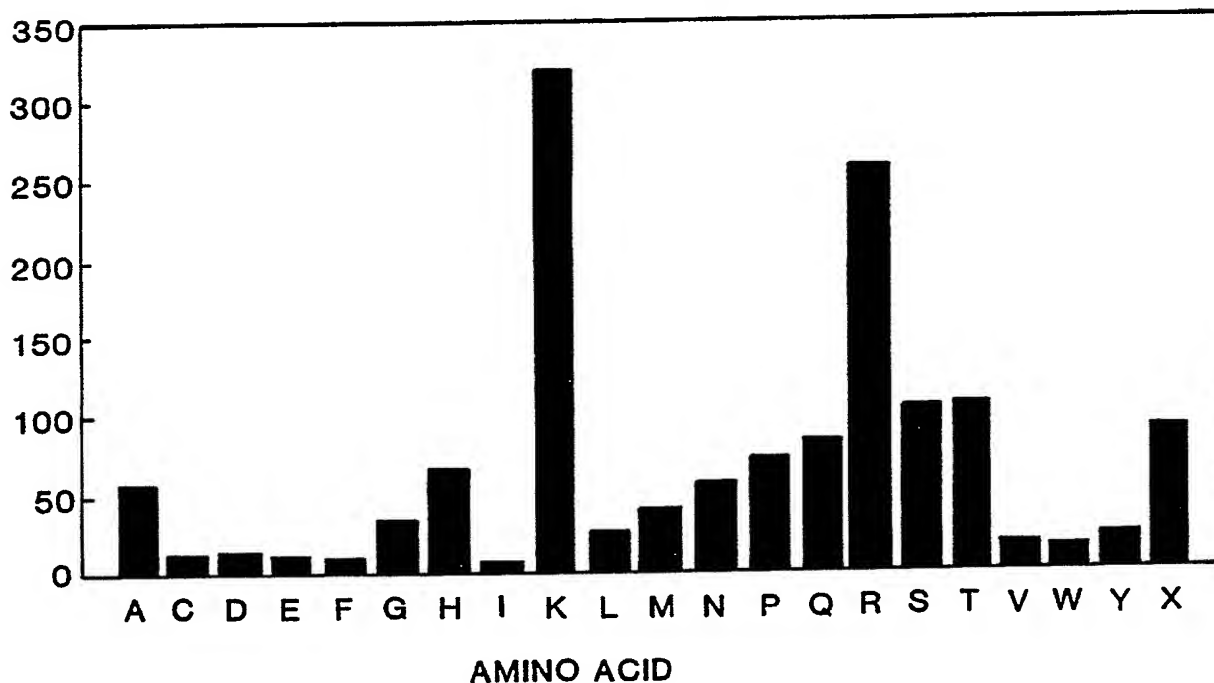
2 / 4

**Ac-rfwmyo-NH₂**

	nM
Ac-rfwmyr	137
Ac-rfwmyk	279
Ac-rfwmyx	366
Ac-rfwmyh	370
Ac-rfwmys	659
Ac-rfwmyt	697
Ac-rfwmyq	707
Ac-rfwmyp	720
Ac-rfwmya	816
Ac-rfwmyn	833
Ac-rfwmyg	922
Ac-rfwmyy	1044
Ac-rfwmyl	1457
Ac-rfwmym	2281
Ac-rfwmyf	3730
Ac-rfwmyc	3769
Ac-rfwmyw	3800
Ac-rfwmye	4496
Ac-rfwmyd	4980
Ac-rfwmyv	12402
Ac-rfwmyi	53641

FIG. IB

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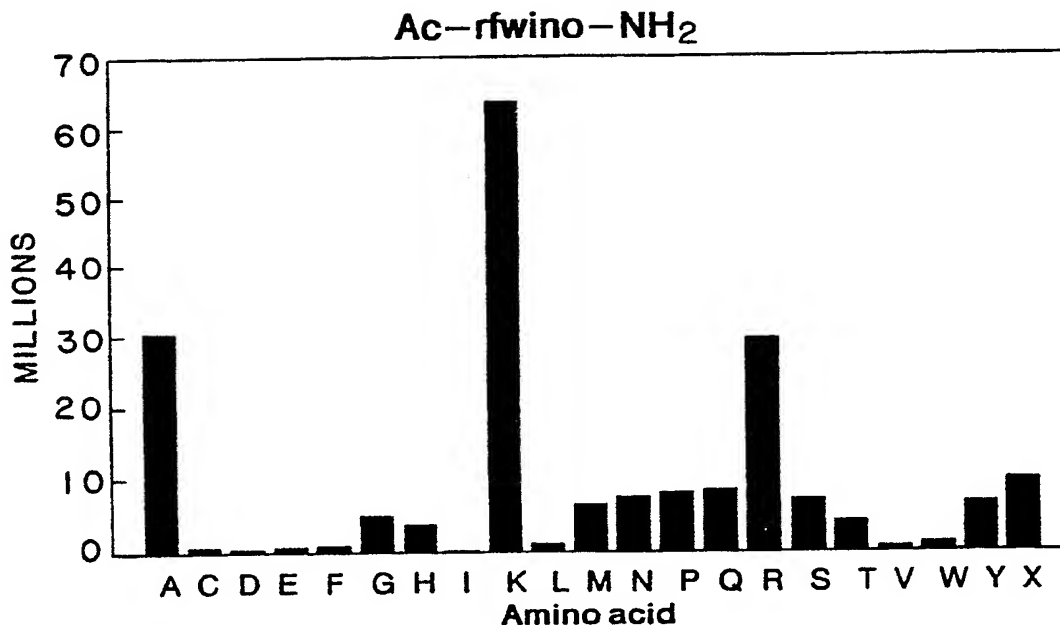
Ac-RFMWMTO-NH₂

Ac-RFMWMTO-NH ₂	nM
Ac-RFMWMTK	3
Ac-RFMWMTR	4
Ac-RFMWMTT	9
Ac-RFMWMTS	10
<u>Ac-RFMWMT</u>	<u>11</u>
Ac-RFMWMTQ	12
Ac-RFMWMTP	14
Ac-RFMWMTH	15
Ac-RFMWMTA	18
Ac-RFMWMTN	18
Ac-RFMWMTM	25
Ac-RFMWMTG	31
Ac-RFMWMTL	40
Ac-RFMWMTY	42
Ac-RFMWMTV	52
Ac-RFMWMTW	63
Ac-RFMWMTD	80
Ac-RFMWMT C	87
Ac-RFMWMT E	93
Ac-RFMWMT F	96
Ac-RFMWMT I	161

FIG. 1C

SUBSTITUTE SHEET (RULE 26)

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**Ac-rfwino-NH₂**

	nM
Ac-rfwink	16
Ac-rfwina	33
Ac-rfwir	34
Ac-rfwinx	103
Ac-rfwinq	120
Ac-rfwinp	125
Ac-rfwinn	137
Ac-rfwins	142
Ac-rfwiny	155
Ac-rfwinnm	159
Ac-rfwing	198
Ac-rfwint	244
Ac-rfwinh	277
Ac-rfwinsw	826
Ac-rfwini	897
Ac-rfwinf	1002
Ac-rfwine	1149
Ac-rfwinc	1311
Ac-rfwinv	1599
Ac-rfwind	1909
Ac-rfwini	8000

FIG. ID**SUBSTITUTE SHEET (RULE 26)**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05616**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :A61K 37/02; C07K 5/00, 7/00, 15/00, 17/00

US CL :530/328, 329

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/328, 329

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPTO APS

search terms: arg-phe-met-trp-met-thr, arg-phe-trp-trp-gly, arg-phe-trp-ile-asn, arg-phe-trp-met-tyr, dooley, houghten

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A, 4,396,606 (GOLDSTEIN) 02 August 1983.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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